

## THE CO<sub>2</sub> SENSITIVITY OF THE HEMOCYANINS AND ITS RELATIONSHIP TO Cl<sup>-</sup> SENSITIVITY

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### ABSTRACT

The effect of CO<sub>2</sub> on hemocyanin-oxygen binding is not generally related to the effect of Cl<sup>-</sup>. Some hemocyanins respond to both and some to either one alone. The direction of the responses of O<sub>2</sub> affinity of the various hemocyanins to CO<sub>2</sub> is poorly correlated with the direction of responses to other effectors. The influence of CO<sub>2</sub> on *Busycon* and *Limulus* hemocyanins reaches its maximum at high pH. Since the effect can be abolished by restoring divalent cation activities to the control levels prior to the addition of CO<sub>2</sub>, we suggest that the effect is not specific but rather indirect, by the pairing of the allosteric effectors Ca<sup>+2</sup> and Mg<sup>+2</sup> with the CO<sub>2</sub> anions. In contrast the effect of CO<sub>2</sub> on crustacean hemocyanins is greater at low pH and it can be enhanced by maintaining HCO<sub>3</sub><sup>-</sup> levels within narrow limits and permitting PCO<sub>2</sub> to vary by a large factor. This finding suggests that the effective species is molecular CO<sub>2</sub>. While Cl<sup>-</sup> influences only oxygen affinity, CO<sub>2</sub> may influence cooperativity as well. In different species the effects of both Cl<sup>-</sup> and CO<sub>2</sub> may or may not be great enough to be physiologically important.

### INTRODUCTION

A site on the hemocyanin (Hc) molecule which is linked to the active site competitively binds divalent cations and H<sup>+</sup>. Its molecular features and their physiological importance have been explored in detail (Arisaka and Van Holde, 1979; reviewed by Mangum, 1980; Miller and Van Holde, 1981).

The nature and significance of anion binding to the Hcs are less clear. While the effects of the organic anion L-lactate on the crustacean Hcs are beginning to be elucidated by a number of investigators (reviewed by Bridges and Morris, 1986), the influence of Cl<sup>-</sup> is not as well understood. Cl<sup>-</sup> specifically influences HcO<sub>2</sub> binding in the chelicerate arthropod *Limulus* (Sullivan *et al.*, 1974; Diefenbach and Mangum, 1983) and the gastropod mollusc *Busycon* (Mangum and Lykkeboe, 1979), but its effect on crustacean Hcs ranges from strong (Brouwer *et al.*, 1978) to absent (Truchot, 1975; Mason *et al.*, 1983). Brix and Torensma (1981) and Torensma and Brix (1981) concluded that the effects of Cl<sup>-</sup> and CO<sub>2</sub> on gastropod Hcs are both allosteric and linked to one another, suggesting a Cl<sup>-</sup>-HCO<sub>3</sub><sup>-</sup> site analogous to the divalent cation-H<sup>+</sup> site.

In fact, the effects of CO<sub>2</sub> in whatever form are even less clear than those of Cl<sup>-</sup>. As pointed out earlier (Burnett and Infantino, 1984), only one of the several investigations on the subject convincingly demonstrates a significant and specific effect of CO<sub>2</sub> on O<sub>2</sub> affinity of the crustacean Hcs (Truchot, 1973). In contrast, a number of negative reports have appeared (reviewed by Burnett and Infantino, 1984; see also

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Morris and Bridges, 1985). After the paper by Burnett and Infantino (1984) was in press a large specific effect of CO<sub>2</sub> on a crab Hc was reported by Greenaway *et al.* (1983) but, curiously, the response is opposite to that found in *Carcinus* (Truchot, 1973).

An effect of very low levels of CO<sub>2</sub> on O<sub>2</sub> affinity of gastropod Hc has been found in three species (Mangum and Lykkeboe, 1979; Torensma and Brix, 1981) but the mechanism remains unclear. Mangum and Lykkeboe (1979) suggested that it may be either direct and specific, *viz.* by means of CO<sub>2</sub> binding to a site linked to the active site, or indirect and non-specific, *viz.* by means of ion-pair formation of HCO<sub>3</sub><sup>-</sup> and CO<sub>3</sub><sup>-2</sup> with the divalent cations, thus inhibiting the action of other allosteric effectors. Brix and Torensma (1981) and Torensma and Brix (1981) described the CO<sub>2</sub> effect as allosteric, but presented as supporting evidence only the fit of their data to the Monod-Wyman-Changeux model. However, the model, which describes a particular form of allosteric behavior, would not distinguish between direct and indirect allosteric actions.

We have investigated the effect of CO<sub>2</sub> and Cl<sup>-</sup> on HcO<sub>2</sub> binding in a variety of species chosen because they are either the same as or closely related to those studied earlier and also because they may represent various combinations of CO<sub>2</sub> and Cl<sup>-</sup> sensitivity. We have investigated the mechanisms of CO<sub>2</sub> effects on gastropod (and also chelicerate) Hcs, by controlling the levels of divalent cation activity while varying those of CO<sub>2</sub> anions. If the divalent cations are the immediate effectors, we would expect to see no effect of CO<sub>2</sub>. We have investigated the mechanism of the effect on crustacean Hcs by controlling (in large part) the levels of the CO<sub>2</sub> anions while allowing CO<sub>2</sub> to vary by a large factor. In this case we would expect the CO<sub>2</sub> effect to vary if molecular CO<sub>2</sub> is the immediate effector.

## MATERIALS AND METHODS

### *Collection and holding of animals*

The crustaceans *Callinectes sapidus* Rathbun and *Palaeomonetes pugio* Holtuis, the chelicerate *Limulus polyphemus* (Linnaeus), and the gastropod mollusc *Busycon canaliculatum* Linnaeus were collected locally. The crustacean *Penaeus duorarum* Burkenroad was collected offshore of Beaufort, North Carolina and *Carcinus maenas* (Linnaeus) at Mt. Desert Island, Maine. The polyplacophoran mollusc *Cryptochiton stelleri* Middendorff was purchased from commercial sources. Animals were held at 16–25°C, depending on origin, and salinities (20–35‰) either identical to those at the collection sites or approximating those specified in previous investigations. The experimental temperature was chosen similarly.

### *Procurement and preparation of samples*

If the ionic composition of the blood of a species was unknown it was determined with ion-selective electrodes (Mangum and Lykkeboe, 1979).

Blood samples were obtained by syringe sampling. The samples were centrifuged to remove debris, in the case of the arthropods after first dec clotting with a tissue grinder. The sera were then either used for O<sub>2</sub> binding measurements without modification or first dialyzed against the desired saline (4°C, 24 h).

### *O<sub>2</sub> binding*

O<sub>2</sub> binding was determined by two methods: (1) for measurements in the presence of different levels of CO<sub>2</sub> a spectrophotometric method was employed (Burnett, 1979;

Burnett and Infantino, 1984). Mixtures of  $N_2$  (99.9995%, scrubbed with Oxisorb),  $CO_2$  (99.5%), and, depending on  $PO_2$ , either air (scrubbed with soda lime and Dri-Rite) or  $O_2$  (99.999%) were prepared with Wosthoff pumps, humidified in a gas washing bottle, and passed over samples incubated in a thermostatically controlled shaker bath. The samples had been diluted by factors ranging from 13.5 to 101 with 0.05 M Tris Maleate buffered saline. Changes in absorbance (1 cm light path) were determined at 335–345 nm, depending on species, with a Bausch & Lomb Spectronic 20 colorimeter. These data are illustrated with open symbols.

(2) The cell respiration method (Mangum and Lykkeboe, 1979) was used to determine the effects of inorganic ions. Samples were diluted by 10% with buffered saline or buffered test solution (final concentration 0.05 M Tris Maleate buffer), and pH (Fisher Accumet with Ross electrode) and  $PCO_2$  (Radiometer electrode, calibrated with 1.05 and 3.03%  $CO_2$ ) were measured at the end of an experiment. These data are illustrated with closed symbols. In this procedure  $PO_2$  is lowered at a constant rate by respiring yeast cells, which also excrete  $CO_2$ . To ascertain that  $PCO_2$  in a buffered preparation does not change during an experiment, a second measurement was performed in parallel, but  $PCO_2$  was measured instead of  $PO_2$ . Following equilibration of the electrode with the sample, no change was detected (Fig. 1).

### *O<sub>2</sub> carrying capacity*

The total  $O_2$  capacity of polyplacophoran blood is known from a single observation on a single individual (Redmond, 1962). After first subtracting the absorbance of deoxyHc to eliminate light scatter, an estimate was made from the spectrophotometric data using the extinction coefficient for gastropod Hc (Nickerson and Van Holde, 1970). Total  $O_2$  was also obtained from the records made during the cell respiration procedure as follows (Fig. 2): The area under the (dashed) line representing a constant rate of  $O_2$  depletion describes the volume of free  $O_2$ , which can be evaluated knowing

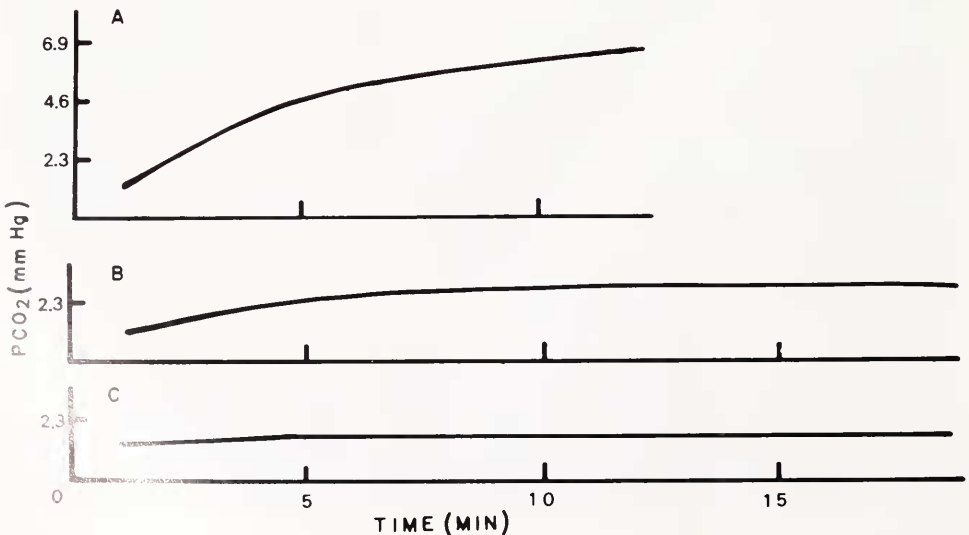


FIGURE 1. Recording of  $PCO_2$  during depletion of  $O_2$  in: A. Saline, with no Hc and no exogenous buffer. B. Saline + *Penaeus duorarum* Hc but no exogenous buffer. C. Saline + *P. duorarum* Hc and 0.05 M Tris Maleate buffer (pH 7.6). 20°C.

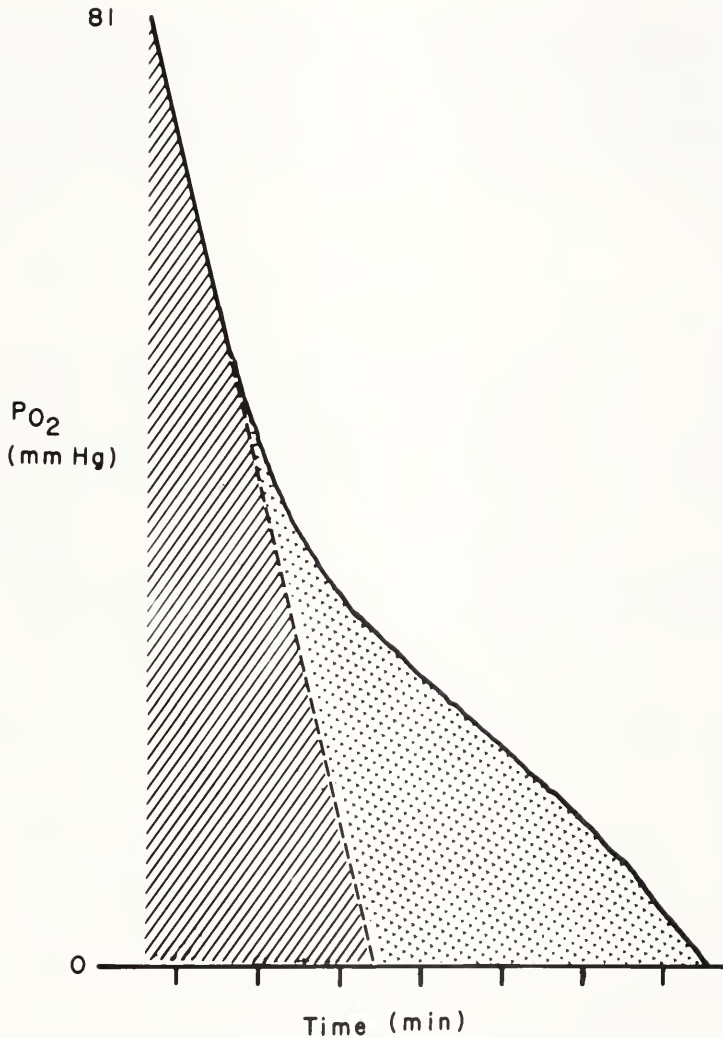


FIGURE 2. O<sub>2</sub> depletion by yeast cells in presence of *Cryptochiton stelleri* Hc. pH 7.43, 15°C. Hatched area shows volume of free O<sub>2</sub>; stippled area shows volume of HcO<sub>2</sub>.

temperature and solute concentration. The area under the curve representing the deviation from that line describes the volume of Hc-bound O<sub>2</sub>, which can be evaluated as a fraction or, more often, a multiple of the free O<sub>2</sub> area.

#### Data analysis

When pH, CO<sub>2</sub>, and Cl<sup>-</sup> were either controlled or when all three had no detectable effect, the significance of differences between mean values of P<sub>50</sub> or n<sub>50</sub> was estimated by Student's *t*-test; the probability of a significant difference is specified below. When pH and CO<sub>2</sub> were varied simultaneously and Cl<sup>-</sup> controlled, the data were described by semilogarithmic (log Y) regression lines and the overlap (if any) between 95% confidence intervals around the lines used as the criterion of significance. When NaCl

was varied while pH and  $\text{PCO}_2$  were controlled, the  $P_{50}$  data were described by semi-logarithmic regression lines and the difference of the slope from zero (at  $P = .05$ ) used as the criterion. Finally, when  $\text{HCO}_3^-$  and NaCl were controlled while pH and  $\text{PCO}_2$  were varied simultaneously, the data were described by semilogarithmic regression lines and the overlap (if any) between 95% confidence intervals around the slopes was used as the criterion. If a substance is said below to have an effect these criteria were exceeded.

## RESULTS

### *Busycon canaliculatum*

Mangum and Lykkeboe (1979) showed that in the channeled conch  $\text{Cl}^-$  has a specific effect on  $\text{HcO}_2$  binding and that, at high pH, the addition of molecular  $\text{CO}_2$  lowers the oxygen affinity of low salinity samples and raises the oxygen affinity of high salinity samples, but only above pH 7.9 where the reverse Bohr shift is quenched by divalent cations. They suggested that, if the  $\text{CO}_2$  effect were indirect (*viz.* by means of ion-pair formation with the divalent cations), the difference between high and low salinity could be explained quantitatively by their findings on the effects of individual inorganic ions: at high salinity the addition of  $\text{CO}_2$  immobilizes some of the divalent cations that normally lower oxygen affinity but enough  $\text{Ca}^{+2}$  and  $\text{Mg}^{+2}$  remains to quench the reverse Bohr shift; the net result is an increase in oxygen affinity. At low salinity, where  $\text{Ca}^{+2}$  and  $\text{Mg}^{+2}$  are already scarce, their immobilization by  $\text{CO}_2$  does not leave enough free divalent cations to quench the reverse Bohr shift.  $\text{Na}^+$  and  $\text{Cl}^-$  become the dominant inorganic ions, and they lower oxygen affinity, which is in fact the net result. The present findings confirm the effect of  $\text{CO}_2$  at high salinity, only above about pH 7.9 (Fig. 3).

To more directly demonstrate the mechanism the following experiment was performed: first a sample of serum was dialyzed against buffered physiological saline for 24 h and  $\text{O}_2$  binding determined. Then 25 mM  $\text{NaHCO}_3^-$  was added to the stirred preparation in which a  $\text{Ca}^{+2}$  selective electrode was immersed, and the change in Ca activity noted.  $\text{O}_2$  binding was determined again. While the sample was stirred with the Ca electrode immersed,  $\text{Ca}(\text{NO}_3)_2$  was then added in sufficient quantity to restore the original level of Ca activity;  $\text{O}_2$  binding was measured a third time. This experiment controls for pair-formation of the  $\text{CO}_2$  anions with  $\text{Ca}^{+2}$  but not  $\text{Mg}^{+2}$ . It could not be performed with a total divalent cation selective electrode for a variety of reasons, the most important of which are the lower sensitivity and resolution of the electrode. Normally these problems are mitigated by chelating Ca with EGTA and then measuring pMg alone; the remedy would not have been possible in the present context without eliminating an important effector of  $\text{O}_2$  binding. Since the percent pair formation of Ca and Mg with the  $\text{CO}_2$  anions is the same (Kester and Pytkowicz, 1969) and since the effects of  $\text{Ca}^{+2}$  and  $\text{Mg}^{+2}$  on  $\text{O}_2$  binding are very nearly so (Mangum and Lykkeboe, 1979), the pair formation with Mg was calculated and additional  $\text{Ca}(\text{NO}_3)_2$  was added to simulate the original  $\text{Mg}^{+2}$  activity using  $\text{Ca}^{+2}$  instead. Changes in monovalent anions of those small magnitudes have no detectable effect (Mangum and Lykkeboe, 1979).  $\text{O}_2$  binding was determined a fourth time.

As reported earlier (Mangum and Lykkeboe, 1979), the addition of  $\text{NaHCO}_3$  to the blood raises the  $\text{O}_2$  affinity of high salinity blood and lowers the  $\text{O}_2$  affinity of low salinity blood (Table I). It does not clearly influence cooperativity ( $P = .09-0.70$ ). The third and fourth steps of the experiment were performed on high salinity blood. When the original activity of only Ca was restored,  $\text{O}_2$  affinity dropped slightly though sig-

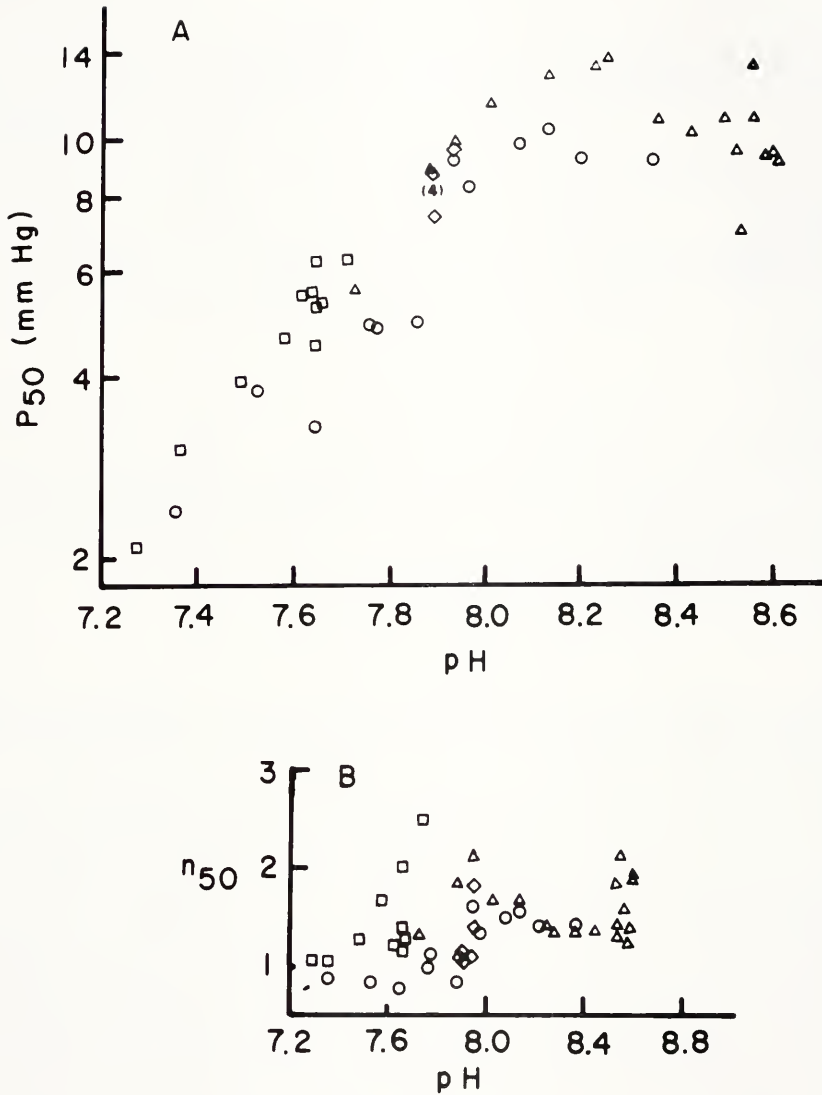


FIGURE 3. Effect of CO<sub>2</sub> on *Busycon canaliculatum* HcO<sub>2</sub> binding. Tonometric method. (○) PCO<sub>2</sub> = 0; (△) PCO<sub>2</sub> = 0.74 mm Hg; (◇) PCO<sub>2</sub> = 7.4 mm Hg; (□) PCO<sub>2</sub> = 14.8 mm Hg. 20°C. Serum was diluted with 0.05 M Tris maleate buffered high salinity saline (from Mangum and Lykkeboe, 1979). A. Oxygen affinity. B. Cooperativity.

nificantly, but still differed from the control value. When additional Ca was added to simulate the change in total divalent cations, O<sub>2</sub> affinity decreased further to a value that is indistinguishable from the control.

#### *Cryptochiton stelleri*

The Hc of the giant sea cradle is clearly cooperative and it has a small (though significant) normal Bohr shift (Fig. 4). The present results essentially agree with earlier

TABLE I

*Effect of NaHCO<sub>3</sub> and Ca(NO<sub>3</sub>)<sub>2</sub> on Busycon canaliculatum HcO<sub>2</sub> binding<sup>1</sup>*

	HS		LS	
	P <sub>50</sub>	n <sub>50</sub>	P <sub>50</sub>	n <sub>50</sub>
Control. PCO <sub>2</sub> 1.50–1.59 mm Hg	10.75 ± 0.31 (8)	1.31 ± 0.04 (8)	9.70 ± 0.40 (6)	1.36 ± 0.13 (6)
+ 25 mM NaHCO <sub>3</sub> PCO <sub>2</sub> 5.5–5.7 mm Hg	9.31 ± 0.17 (6) <sup>2</sup>	1.40 ± 0.03 (6)	10.70 ± 0.30 (8)	1.43 ± 0.25 (8)
Same, + Ca <sup>+2</sup> to Equal pCa in control. PCO <sub>2</sub> 5.7 mm Hg	9.95 ± 0.20 (9) <sup>3</sup>	1.33 ± 0.05 (9)	—	—
Same, + Ca <sup>+2</sup> to Equal pCa + pMg in control	10.72 ± 0.20 (8) <sup>4</sup>	1.33 ± 0.06 (8)	—	—

<sup>1</sup> Cell respiration method: serum was dialyzed against 0.05 M Tris maleate buffered high (HS) or low (LS) salinity saline (from Mangum and Lykkeboe, 1979). 19.7–20.1°C, pH 8.18–8.23. Mean ± S.E. (N).

<sup>2</sup>  $P = 0.0035$  vs control.

<sup>3</sup>  $P = 0.025$  vs control, 0.0375 vs + NaHCO<sub>3</sub> alone.

<sup>4</sup>  $P = 0.04$  vs + Ca<sup>+2</sup> to equal pCa in control, 0.97 vs control.

findings (Manwell, 1958), although the comparison is made somewhat difficult by the different pH ranges investigated. The slope of a regression line describing the two sets of control data for P<sub>50</sub> in Figure 4A is  $-0.20 \pm 0.04$  (95% C. I.). Also in the combined control data, there is a small but significant increase in cooperativity with pH.

The O<sub>2</sub> affinity data for 0 and 14.8 mm Hg PCO<sub>2</sub> differ significantly in the pH range 7.2–7.6 but not in the range 7.7–7.85 (Fig. 4A). At high PCO<sub>2</sub> cooperativity is lower ( $P = 0.002$ ) and there is a small but significant decrease with pH. Neither HcO<sub>2</sub> affinity (Fig. 4B) nor cooperativity ( $1.87 \pm 0.09$  S. E.,  $N = 6$ ) respond to NaCl.

The data in Figure 4 were obtained using blood taken from the hemocoel directly into a hypodermic needle passed through two adjacent dorsal shell plates. When an initial attempt to perform the operation was unsuccessful, a blood sample was obtained by slitting the foot and draining the hemocoel. No other organs were damaged although some mucus was produced. The sample formed a blue precipitate and, after centrifugation at low speed, the supernatant fluid did not combine reversibly with oxygen. When the pellet was washed with saline, it did not go into solution but it still combined reversibly with oxygen. Measured with the cell respiration technique, oxygen affinity was higher than that of the syringe sample, but only by about 25%; cooperativity was lower but still easily detected ( $n_{50} = 1.72 \pm 0.19$  S. E.,  $N = 12$ ). Apparently the native higher order structure is not critical to cooperativity and a relatively low O<sub>2</sub> affinity.

The difference in absorbance between oxygenated and deoxygenated samples, corrected for the dilution factor indicates that the native Hc concentration in one animal was about 0.74 g/100 ml, yielding an HcO<sub>2</sub> capacity of only about 0.33 (total O<sub>2</sub> capacity = 0.91) ml/100 ml. The integrals obtained from the records made during the cell respiration procedure indicate an HcO<sub>2</sub> carrying capacity in another animal of 0.22 ml/100 ml or a total blood O<sub>2</sub> carrying capacity of 0.80 ml/100 ml. The results support the inference of an extremely low O<sub>2</sub> carrying capacity of the blood in this class (Redmond, 1962).

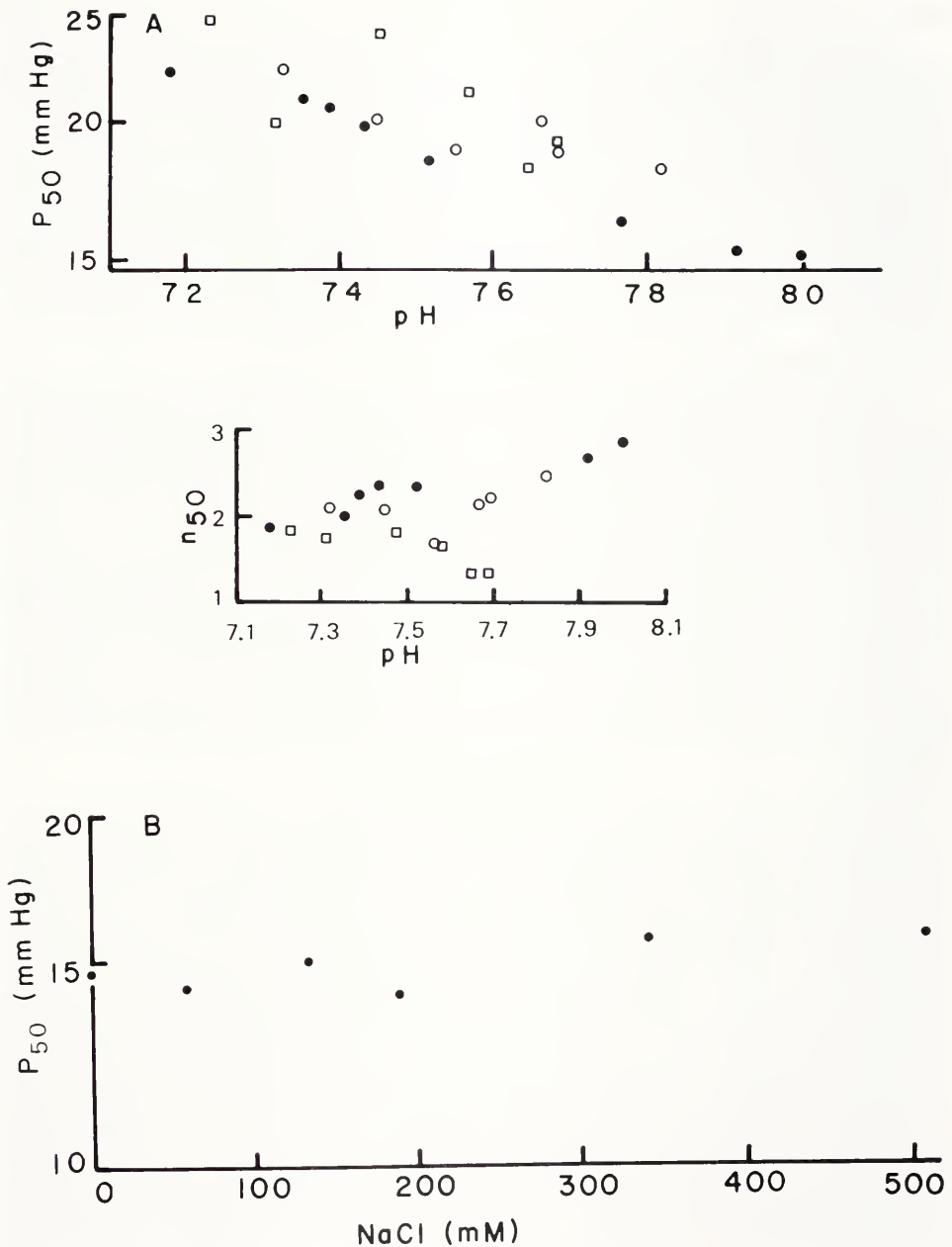


FIGURE 4. A. Effect of CO<sub>2</sub> on *Cryptochiton stelleri* HcO<sub>2</sub> binding. Tonometric method: serum was diluted with 0.05 M Tris maleate buffered, filtered seawater (35‰) in which the animals had been shipped. (○) PCO<sub>2</sub> = 0, (□) PCO<sub>2</sub> = 14.8 mm Hg. 15°C. Cell respiration method: after dialysis against seawater, Tris maleate buffer (final concentration 0.05 M) was added to the serum. (●) PCO<sub>2</sub> 1.32–5.41 mm Hg. B. Effect of NaCl on HcO<sub>2</sub> affinity. Cell respiration method: serum was dialyzed against 10 mM Ca(NO<sub>3</sub>)<sub>2</sub> + 0.05 M Tris maleate buffer, pH 7.62–7.65. PCO<sub>2</sub> 3.0–3.3 mm Hg. 15°C.



*Penaeus duorarum*

At pH 7.6, which is likely to approximate the physiological value, the Hc of the pink shrimp has a moderate oxygen affinity and pronounced cooperativity. In the pH range studied it also has a large normal Bohr shift (Fig. 5); the slope of a regression line describing the two sets of control data is  $-1.09 \pm 0.19$  (95% C. I.). The data are virtually indistinguishable from those reported earlier for *P. setiferus* (Brouwer *et al.*, 1978). As also in *P. setiferus* (Brouwer *et al.*, 1978), *P. duorarum* HcO<sub>2</sub> affinity clearly responds to NaCl (Fig. 5B). However, it responds identically to NaNO<sub>3</sub>, Na<sub>2</sub>SO<sub>4</sub> (plotted as mM cation), and choline Cl<sup>-</sup>. There is a significant decrease in cooperativity ( $P = 0.05-0.001$ , Fig. 5B). Neither property responds to CO<sub>2</sub>, although the trend in cooperativity is the same as that in the data for CO<sub>2</sub> sensitive Hcs ( $P = 0.15$ , Fig. 5A).

*Palaeomonetes pugio*

At putative physiological pH the Hc of the grass shrimp has an extremely low O<sub>2</sub> affinity and fairly little cooperativity (Fig. 6). The slope of a semilogarithmic regression line describing the control data for P<sub>50</sub> is  $-1.33 (\pm 0.56$  95% C. I.). HcO<sub>2</sub> affinity clearly responds to CO<sub>2</sub> (Fig. 6A), which does not appear to influence cooperativity ( $P = 0.18$ ). The pH dependence of P<sub>50</sub> at high PCO<sub>2</sub> appears to be about the same as that at low PCO<sub>2</sub>, but the estimate made by regression analysis is much greater ( $-1.75 \pm 0.11$ ). In view of the far greater variability in the control data ( $r^2 = 0.756$ ) the numerical estimate of the Bohr shift at high PCO<sub>2</sub> ( $r^2 = 0.998$ ), which shows extremely great pH dependence, is probably the more accurate of the two. NaCl clearly raises HcO<sub>2</sub> affinity but does not change cooperativity (Fig. 6B). Due to the great difficulty of obtaining ample volumes of blood from these always small animals, which were even smaller than usual at the time of the experiment on NaCl sensitivity because the population had just reproduced, we did not investigate the specificity of this response.

*Callinectes sapidus*

The Hc of the blue crab does not respond specifically to Cl<sup>-</sup> (Mason *et al.*, 1983). Oxygen affinity (but not cooperativity) does respond to CO<sub>2</sub> (Fig. 7). Because it was concluded earlier that the Hc of a species belonging to the same genus (*C. bellicosus*) is not sensitive to CO<sub>2</sub> within the range 1.5–7.4 mm Hg, the response of *C. sapidus* was examined in more detail. While there may appear to be little or no difference in P<sub>50</sub> at 0 and 1.5 mm Hg (Fig. 7), the two sets of data are in fact significantly different. When the data for *C. bellicosus* are analyzed similarly, however, an effect of CO<sub>2</sub> can be demonstrated only at 23°C and only at pH 7.5 and above (Burnett and Infantino, 1984). Of the other four species examined by Burnett and Infantino (1984) a similar trend may be present in the data for *Pachygrapsus crassipes* (though  $P > .05$ ); however, no sign of an effect can be perceived in the remaining three (Burnett and Infantino, 1984). Thus we suggest that our original conclusion, *viz.* that CO<sub>2</sub> is not an important effector of HCO<sub>2</sub> binding in the five species, was essentially correct (Burnett and Infantino, 1984). Regardless, only at much higher PCO<sub>2</sub> is there an appreciable response of HCO<sub>2</sub> affinity in *C. sapidus*; cooperativity does not respond even at that level (Fig. 7).

In an attempt to identify the CO<sub>2</sub> species responsible for the effect, we dialyzed aliquots of a sample against either (1) saline containing no exogenous CO<sub>2</sub>, or (2) saline containing high levels of NaHCO<sub>3</sub>. When pH was adjusted by adding Tris maleate buffered saline (final concentration 0.05 M), PCO<sub>2</sub> varied by more than an order of magnitude while HCO<sub>3</sub><sup>-</sup> varied by only about 25% (Fig. 7B). The oxygen affinity of this sample was depressed even more at low pH and high PCO<sub>3</sub> than at high pH and low PCO<sub>2</sub>. If this result were due to the greater change in pCa at high

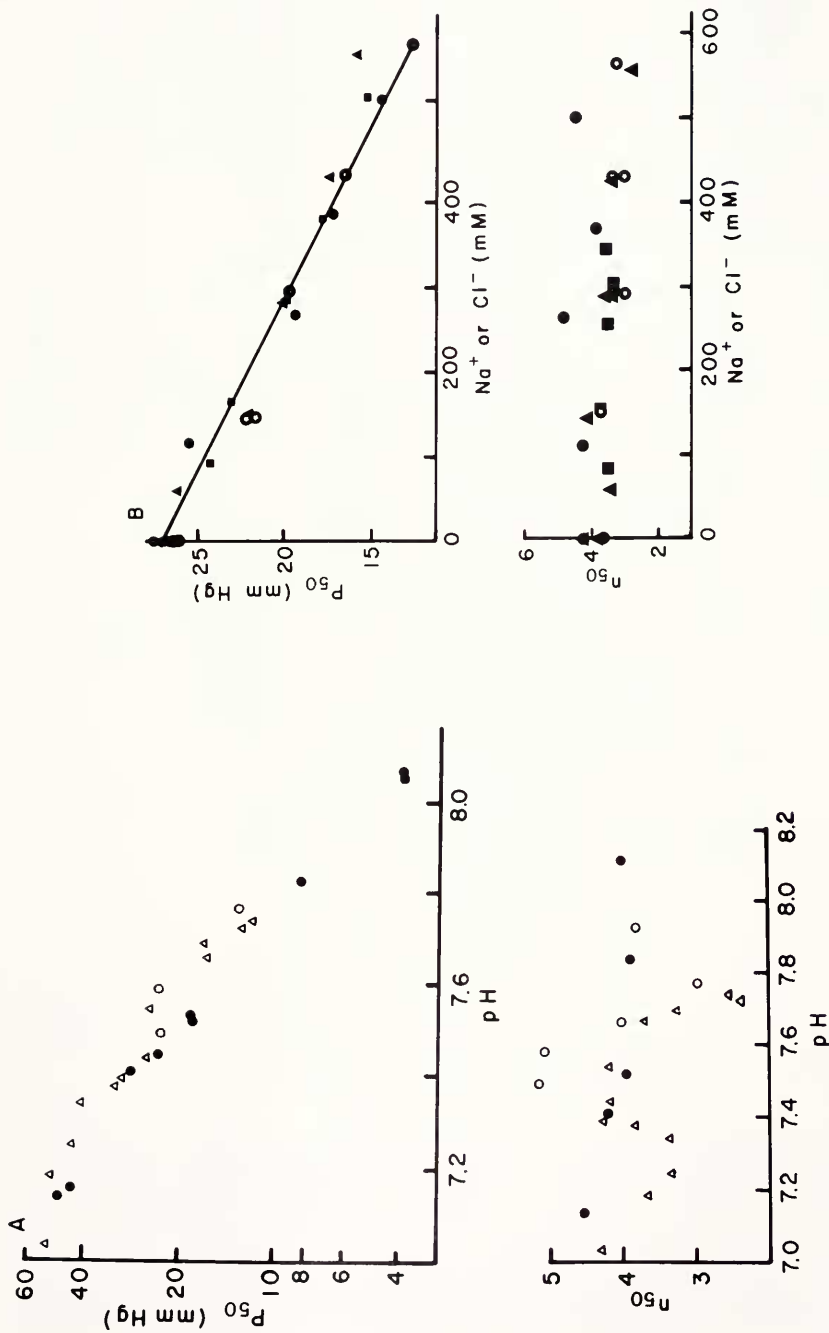


FIGURE 5. A. Effect of CO<sub>2</sub> on *Penaeus duorarum* HcO<sub>2</sub> binding. Tonometric method: serum was diluted with 0.05 M Tris maleate buffered saline containing 11 mM KCl, 14 mM CaCl<sub>2</sub>, 39 mM MgCl<sub>2</sub>, 433 mM NaCl, and 32 mM Na<sub>2</sub>SO<sub>4</sub>. (○) PCO<sub>2</sub> = 0, (△) PCO<sub>2</sub> = 14.8 mm Hg. Cell respiration method: serum was dialyzed against buffered saline described above, PCO<sub>2</sub> = 1.30–6.60 mm Hg. B. Effect of (●) NaCl, (■) NaNO<sub>3</sub>, (▲) Na<sub>2</sub>SO<sub>4</sub>, and (○) choline Cl<sup>-</sup>. Cell respiration method: serum was dialyzed against 0.05 M Tris maleate buffered Ca(NO<sub>3</sub>)<sub>2</sub> (15 mM, pH 7.58–7.69, PCO<sub>2</sub> = 2.0–3.3 mm Hg), 20°C.

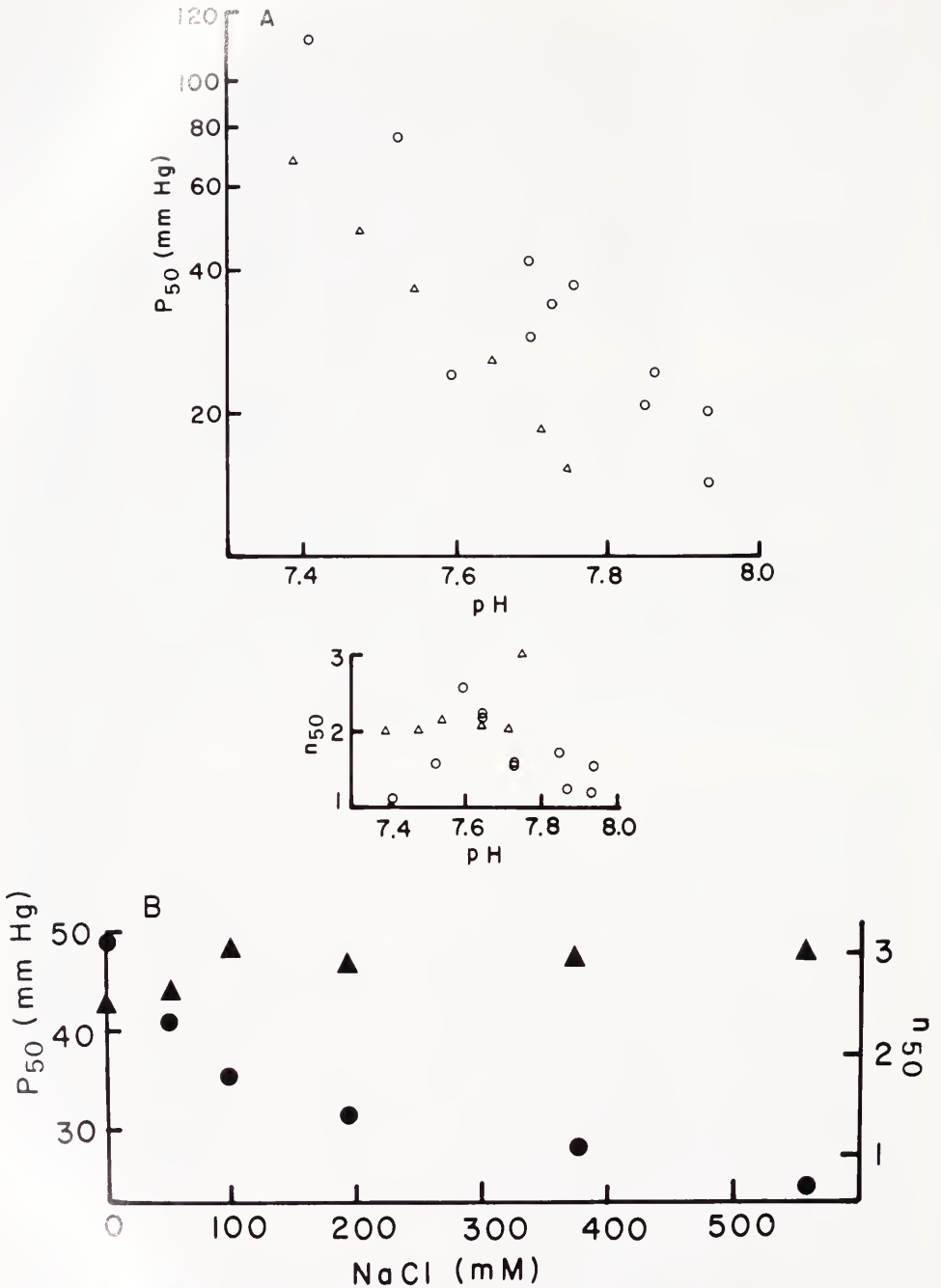


FIGURE 1. A. Effect of CO<sub>2</sub> on *Palaemonetes pugio* HcO<sub>2</sub> binding. Tonometric method: serum was dialyzed against a saline containing 261 mM NaCl, 8.1 mM KCl, 6.3 mM CaCl<sub>2</sub>, 4.4 mM MgCl<sub>2</sub>, and 29.5 mM Na<sub>2</sub>SO<sub>4</sub> (from Mantel and Farmer, 1983), and diluted with 0.05 M Tris maleate buffered saline. (○) PCO<sub>2</sub> = 0, (Δ) PCO<sub>2</sub> = 14.8 mm Hg, 17°C. B. Effect of NaCl on HcO<sub>2</sub> binding. Cell respiration method: serum was dialyzed against 10 mM Ca(NO<sub>3</sub>)<sub>2</sub> + 0.05 mM Tris maleate buffer, pH 7.65–7.68, PCO<sub>2</sub> 1.3–1.5 mm Hg, 25°C. (●) P<sub>50</sub>, (▲) n<sub>50</sub>.

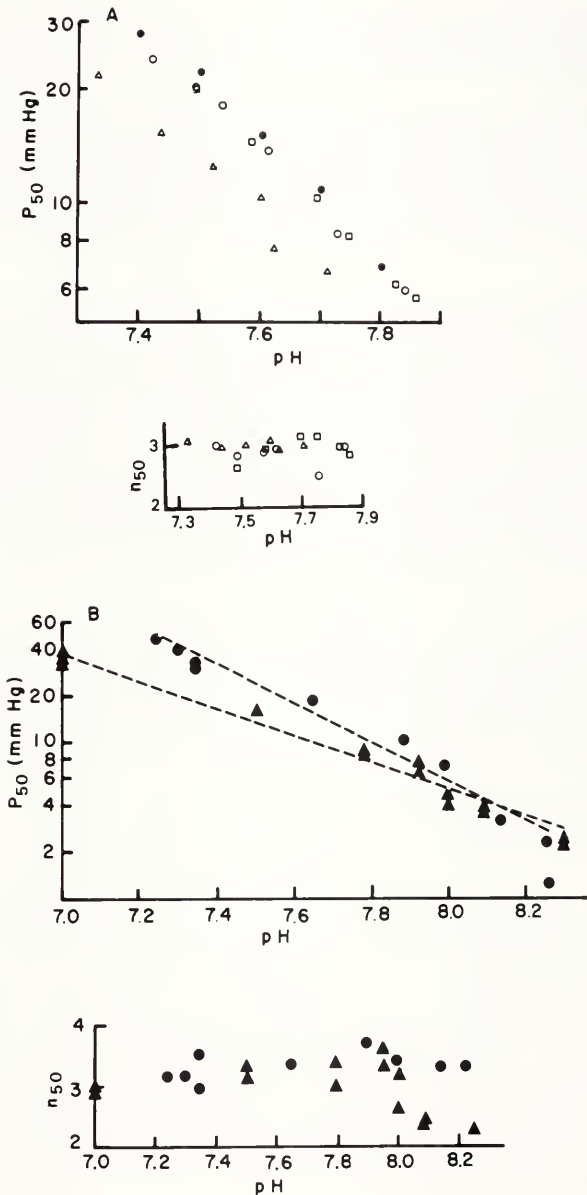


FIGURE 7. A. Effect of CO<sub>2</sub> on *Callinectes sapidus* HcO<sub>2</sub> binding. Tonometric method: serum was diluted with 0.5 M Tris maleate buffered high salinity saline (from Mason *et al.*, 1983). 25°C. (○) PCO<sub>2</sub> = 0, (□) PCO<sub>2</sub> = 1.5 mm Hg; (△) PCO<sub>2</sub> = 14.8 mm Hg. (●) For comparison, unpublished data collected earlier by cell respiration method: serum was dialyzed against same Tris maleate buffered saline. 25°C. PCO<sub>2</sub> unknown. B. Effect of high NaHCO<sub>3</sub>. Cell respiration method: in controls (●), PCO<sub>2</sub> and [NaHCO<sub>3</sub>] vary from 0.32 mm Hg and 4.09 mM at pH 8.25 to 6.98 mm Hg and 1.69 mM at pH 7.24. In experiments (▲), PCO<sub>2</sub> and [NaHCO<sub>3</sub>] vary from 1.2 mm Hg and 13.7 mM at pH 8.25 to 1.4 mm Hg and 10.61 mM at pH 7.00. 25°C. Dashed lines, which differ significantly, were fitted by regression analysis.

than at low pH.  $O_2$  affinity should have been lowered at high pH and unchanged at low pH rather than the other way around.

### *Carcinus maenas*

Truchot (1973, 1975) showed earlier that, in European members of this species,  $Cl^-$  has no effect whereas  $CO_2$  clearly raises  $O_2$  affinity. Our data confirm the  $CO_2$  effect, which occurs in about the same magnitude in the North American population (Fig. 8). They also show that  $CO_2$  significantly lowers cooperativity and its pH dependence.

### *Limulus polyphemus*

$Cl^-$  has a specific effect on  $HcO_2$  affinity (but not cooperativity) in the horseshoe crab (Sullivan *et al.*, 1974; Diefenbach and Mangum, 1983).  $CO_2$  has a significant effect on  $O_2$  affinity as well; an apparent decrease in cooperativity is not quite significant ( $P = 0.11$ ; Fig. 9). The experiment designed to identify the mechanism of the  $CO_2$  effect on *Busycon* Hc was also performed on *Limulus* Hc, with the exception that the step simulating the effect of changing only pCa was omitted. The addition of  $NaHCO_3$  significantly raises  $O_2$  affinity (Table II) but not cooperativity ( $P = 0.19$ ) and the addition of  $Ca(NO_3)_2$  to restore the original levels of free  $Ca^{+2}$  and  $Mg^{+2}$  lowers  $O_2$  affinity back again to a value that does not differ from the control.

## DISCUSSION

Various combinations of  $Cl^-$  and  $CO_2$  sensitivity of the Hcs can be found. In any particular species  $HcO_2$  affinity may be sensitive to both (*e.g.*, *Busycon*, *Limulus*, and *Palaeomonetes*), to  $Cl^-$  but not  $CO_2$  (*Penaeus*) or to  $CO_2$  but not  $Cl^-$  (*Cryptochiton*,

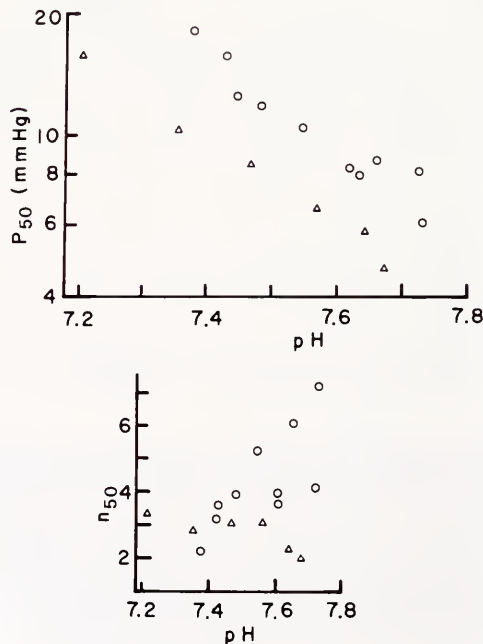


FIGURE 8. Effect of  $CO_2$  on *Carcinus maenas*  $HcO_2$  binding. Tonometric method: serum was diluted with 0.05 M Tris maleate buffered saline (from Robertson, 1960). 16°C, (○)  $PCO_2 = 0$ , (△)  $PCO_2 = 14.8$  mm Hg.

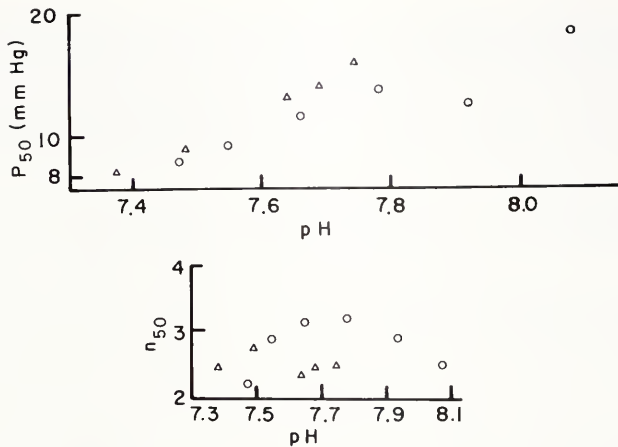


FIGURE 9. Effect of CO<sub>2</sub> on *Limulus polyphemus* HcO<sub>2</sub> binding. Tonometric method: serum was diluted with 0.05 M Tris maleate buffered high salinity saline (from Towle and Mangum, 1982). (○) PCO<sub>2</sub> = 0, (△) PCO<sub>2</sub> = 14.8 mm Hg, 25°C.

*Carcinus*, and *Callinectes*). In contrast the effect of CO<sub>2</sub> (if it occurs) is poorly correlated with sensitivity to other ionic effectors. While CO<sub>2</sub> consistently raises the O<sub>2</sub> affinity of Hcs (*Busycon*, *Limulus*) with reverse Bohr shifts (and, more importantly, positive divalent cation responses), it may either raise (*Callinectes*, *Carcinus*, and *Palaeomonetes*), lower (*Cryptochiton*) or have no effect on (*Penaeus*) the O<sub>2</sub> affinity of Hcs with normal Bohr shifts. Different combinations of CO<sub>2</sub> sensitivity of HcO<sub>2</sub> affinity and cooperativity can also be found. CO<sub>2</sub> may affect O<sub>2</sub> affinity but not cooperativity (e.g., *Busycon*, *Limulus*, and *Callinectes*), both (*Carcinus*, *Cryptochiton*, and perhaps *Palaeomonetes*) or perhaps neither (*Penaeus*). If CO<sub>2</sub> does have an effect, it generally lowers cooperativity and reduces its pH dependence.

Our results suggest that the chemical species responsible for the effect of CO<sub>2</sub> on portunid crab HcO<sub>2</sub> affinity is molecular carbon dioxide. The effect is greater at low than at high pH and the effector changes O<sub>2</sub> affinity in the same direction as the divalent cations. We can think of no indirect mechanism that would produce such a response. Moreover, the experiment in which HCO<sub>3</sub><sup>-</sup> was held within a relatively narrow range while PCO<sub>2</sub> was allowed to change by a large factor produced a much greater effect of CO<sub>2</sub> at low pH, also implicating the molecular species.

Our results also support the hypothesis that the mechanism responsible for the effect on *Busycon* and *Limulus* Hcs is the action of the CO<sub>2</sub> anions on the allosteric effectors free Ca<sup>+2</sup> and Mg<sup>+2</sup> rather than a direct effect. It occurs only at high pH, it

TABLE II

Effect of NaHCO<sub>3</sub> and Ca(NO<sub>3</sub>)<sub>2</sub> on *Limulus polyphemus* HcO<sub>2</sub> binding<sup>1</sup>

	P <sub>50</sub>	n <sub>50</sub>
Controls PCO <sub>2</sub> 1.06 mm Hg	17.1 ± 0.2 (5)	1.89 ± 0.03 (5)
+ 25 mM NaHCO <sub>3</sub> PCO <sub>2</sub> 5.05 mm Hg	15.4 ± 0.3 (5) <sup>2</sup>	1.82 ± 0.05 (5)
+ Ca (NO <sub>3</sub> ) <sub>2</sub> to equal pCa + pMg in control	16.9 ± 0.5 (6) <sup>3</sup>	1.38 ± 0.05 (6)

<sup>1</sup> Cell respiration method: serum was dialyzed against 0.05 M Tris maleate buffered high salinity saline (from Towle and Mangum, 1982), 19.7°C. pH 8.21–8.23. Mean ± S.E. (N).

<sup>2</sup> P < 0.001 vs control.

<sup>3</sup> P = 0.05 vs + NaHCO<sub>3</sub>, 0.75 vs control.

requires more  $\text{CO}_2$  at high than at low salinity and it can be quantitatively explained by the restoration of total divalent cation activity to levels that existed prior to the addition of  $\text{CO}_2$ . There is no reason to postulate a binding site linked to the active site for which anions compete. The similar responses in these two species also support the conclusion drawn earlier that the fundamentally different quaternary structures of the molluscan and arthropod Hcs do not mandate different  $\text{O}_2$  binding properties (Mangum *et al.*, 1985).

The surprising effect of  $\text{CO}_2$  on the Hc of the freshwater crab *Holthuisana* (Greenaway, Bonaventura, and Taylor, 1983; Greenaway, Taylor, and Bonaventura, 1983) may be due to the same phenomenon; this Hc may not be directly sensitive to carbon dioxide. Unlike the response of other crustacean Hcs, the  $\text{O}_2$  affinity of this molecule decreases with the addition of molecular  $\text{CO}_2$ , (9.9 mm Hg), and the effect is slightly greater at pH 7.6 than at 7.22–7.30. Moreover, the levels of divalent cations in the blood of this freshwater crab are presumably relatively low (Greenaway and MacMillen, 1978), even at low levels of  $\text{CO}_2$ . On the other hand, relatively few of the  $\text{CO}_2$  anions would be formed by the addition of 9.9 mm Hg  $\text{PCO}_2$  in that pH range (Greenaway, Bonaventura, and Taylor, 1983). Its Hc would have to be especially sensitive to divalent cations.

Typically the slopes of Hill plots of  $\text{HcO}_2$  equilibria increase with oxygenation. Since these data are often collected by a tonometric procedure that involves the stepwise addition of unscrubbed room air, we suggest that at least a fraction of the slope change may result from an increase in  $\text{O}_2$  affinity with  $\text{CO}_2$  rather than oxygenation, especially in species in which cooperativity is not very sensitive to  $\text{CO}_2$ .

The  $\text{Cl}^-$  sensitivity of *Penaeus* Hc may be either (1) specific and mimicked exactly by a specific effect of  $\text{Na}^+$ , at least within the physiological range, or (2) a general effect of ionic strength. The information presently available does not decide the question.

Since  $\text{CO}_2$  sensitivity is not related to the sensitivity of inorganic anions (and since the nature of the binding sites is totally unknown), it is not possible at present to predict the effects of  $\text{CO}_2$  (or  $\text{Cl}^-$ ) on the  $\text{O}_2$  affinity of an unknown Hc. Regrettably, if the knowledge is needed it must be acquired in each case. Since it may be quite large, the effect of  $\text{Cl}^-$  may be an important factor in understanding how a  $\text{HcO}_2$  transport system works. In *Penaeus*, for example, a change from 330 to 560 mM  $\text{Cl}^-$ , well within the physiological range in many crustaceans (though perhaps not in these offshore species), causes a 50% change in  $P_{50}$  (pH 7.6). Though more often small, the  $\text{CO}_2$  effect may become similarly large at 25°C and physiological pH and  $\text{Cl}^-$ , the maximum being 46% in the range 0–14.8 mm Hg (*Palaemonetes*). This is considerably greater than the effect of  $\text{CO}_2$  on mammalian hemoglobin at the same temperature and at 100 mM  $\text{Cl}^-$  and pH 7.4 (calculated from data shown by Imai, 1982). The effect of  $\text{CO}_2$  on Hc cooperativity is probably even more important. At pH 7.6 the maximum is a change of about 100% (*Carcinus*), which could radically alter oxygenation states at both the gill and the tissues. A more quantitative assessment awaits extensive measurements of physiological extremes of  $\text{PCO}_2$  made simultaneously with measurements of  $\text{HcO}_2$  binding and its other determinants.

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